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By

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Introduction

This study was undertaken to investigate whether unconjugated etiocholanolone (etiocholane-3 alpha-ol-17-one), a pyrogenic androgen metabolite, (1) occurs in the plasma of rabbits and dogs after the injection of pyrogen.

Kappas et al. reported that the parenteral administration of etiocholanolone to normal volunteers produced fever (103°F.) associated with chills, myalgias, arthralgias, anorexia, sweating, and headache (1, 2, 3). Kappas demonstrated that the pyrogenicity of etiocholanolone is determined by the 5 beta hydrogen combined with a 3 alpha hydroxyl group of the steroid nucleus (4). He also demonstrated that alteration of this configuration, such as *in vitro* esterification with acetate at the 3 alpha hydroxyl position, abolished pyrogenic action (4). This same inactivation mechanism occurs *in vivo* with glucuronic acid. Bondy et al. reported that unconjugated etiocholanolone occurred in the plasma of two patients with periodic fever at the time of the acute febrile attack (5). The unconjugated compound was not detected during afebrile intervals.

Several investigators have reported methods for the determination of 17-ketosteroids in plasma after acid and enzymatic hydrolysis; but isolation, measurement, and identification of unconjugated etiocholanolone has not been reported (6, 7, 8). The possibility that unconjugated etiocholanolone might

be an endogenous pyrogen which may mediate the pathogenesis of fever was suggested by the fact that it is pyrogenic and has not been demonstrated in normal human plasma, but in the plasma of patients suffering from acute febrile attacks of periodic fever.

Atkins and Wood demonstrated the occurence of an endogenously released pyrogenic substance in the plasma of rabbits at a specific time after the injection of typhoid endotoxin (9). Plasma containing this substance induced a characteristic febrile response when it was injected into normal recipient rabbits. Atkins and Wood demonstrated that this endogenous pyrogen was distinct from the injected endotoxin.

There are similarities between the properties of the endogenous pyrogen found in the serum of rabbits and unconjugated etiocholanolone. Both substances act as pyrogens, and there is suggestive evidence that both may occur and mediate a febrile response in vivo (5, 9). Neither unconjugated etiocholanolone nor the endogenous pyrogen described by Atkins and Wood has been demonstrated in the plasma of normal subjects. Repeated injection of either substance does not produce tolerance to its thermogenic effect (4, 9).

An attempt was made to demonstrate the presence of unconjugated etiocholanolone in plasma known to contain endogenous pyrogen. A method for the isolation and measurement

of unconjugated etiocholanolone is reported. Plasma samples from animals treated with techniques designed to stimulate the release of endogenous pyrogen were analyzed for this steroid.

Materials and Methods

Experimental Animals:

The rabbit and dog were selected since previous investigators demonstrated the occurence of endogenous pyrogen in these animals. Three healthy, male mongrel dogs weighing 22, 26, and 47 kilograms were used. Plasma samples were obtained from six normal laboratory rabbits (four females and two males). [1]

Administration of Endotoxins to Dogs:

The experiments were carried out according to the method of Petersdorf and Bennett (10). Control rectal temperatures were taken for two days before endotoxin administration. Sterile glassware was used throughout the experiment and the endotoxins were injected under sterile conditions. All blood samples were obtained from dogs under pentobarbital anesthesia. A 100 ml. control blood sample was drawn in heparinized syringes from each animal before the administration of endotoxins.

Two endotoxins were used to stimulate the release of endogenous pyrogen in dogs. Formalin-killed Salmonella Typhosa V-58 monovalent endotoxin (500 x 10^6 organisms per ml.) was injected intravenously in doses of 7 - 20 ml., depending on the weight of the animal. Pyromen, a commerdially

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[1] Plasma samples from experimental rabbits were graciously provided by Dr. Elisha Atkins, Department of Medicine, Yale University School of Medicine.

purified Pseudomonas polysaccharide, was injected in doses of 5 - 8 micrograms per kilogram. Temperature elevations of 1 - 3° F. were obtained. Petersdorf and Bennett reported that these doses of typhoid vaccine and Pyromen produced the greatest amount of endogenous pyrogen two hours after their administration (10). Heparinized blood samples of 100 ml. were obtained two to three hours after the administration of the endotoxins. The experiment was repeated in two of the three dogs after intervals of not less than four days. A total of three control samples and eight experimental samples were obtained.

The Administration of Virus Vaccine to Rabbits:

Plasma samples were obtained from rabbits treated according to the protocol of Atkins and Haung (11). Control blood samples were taken from each animal seven days before the vaccine was administered.

Live Newcastle Disease Virus Vaccine (NDV-14) was injected intravenously to stimulate the release of endogenous pyrogen. Sterile technique was maintained throughout the procedure. Heparinized blood samples were obtained four hours after the injection of the NDV-14 vaccine, since Atkins found the greatest activity of endogenous pyrogen at that time. The volumes of control blood samples were limited by the small blood volume of the animal. The animals were exsanguinated when the experimental blood samples were obtained. This resulted in experimental samples of greater volume than

the controls. Six control samples and five experimental samples were taken.

Reagents and Materials:

1) Standard ethanolic solutions of etiocholanolone, androsterone, and dehydroepiandrosterone.

2) Tritium-labelled etiocholanolone, Specific Activity: 5.2μc/μg. Prepared by the Wilzbach technique and purified by paper chromatography to a constant specific activity. [2]

3) 1N NaOH solution.

4) Solvents:
   Redistilled chloroform
   Methanol
   Distilled heptane
   Absolute ethanol

5) 2, 4-dinitrophenylhydrazine, Reagent Grade.

6) Benedict's Reagent: 4.3 grams Copper Sulfate
   43.3 grams Sodium Citrate
   25.0 grams Sodium Carbonate
   diluted with 250 ml. distilled water.

7) Zimmermann Reagents: 2% meta-dinitrobenzene in 95% ethanol
   2.5 N KOH in absolute ethanol

8) Whatman No. 1 chromatography paper, washed 72 hours in methanol: ethyl acetate, 10:1.

9) Petroleum ether, B.P. 90 - 110° C., Reagent Grade

10) Distilled benzene, Reagent Grade.

11) Aluminum oxide, Woelm-nonalkaline.

Separation:

Plasma was separated from the cells within 30 minutes of collection, and frozen until analyzed. The plasma is thawed

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[2] The Wilzbach procedure was performed by the New England Nuclear Corporation, Boston 18, Massachusetts.

at room temperature and poured into a 250 ml. centrifuge tube. One half ml. (approximately 10,000 counts per minute) of tritium-labelled etiocholanolone is added with stirring to the sample tube and to an isotope counting vial. One ml. of 1 N NaOH per 25 ml. of plasma is added to each sample with stirring. The plasma is extracted three times with 100 ml. volumes of chloroform. The extracts are pooled and concentrated by air drying, and applied to washed Whatman No. 1 chromatography paper.

Twenty micrograms of standard etiocholanolone, androsterone, and dehydroepiandrosterone are spotted alternately with the extract spots. A strip three centimeters wide is allowed for each spot. Two extract strips and three standard strips can be conveniently run on each chromatogram. The chromatogram is equilibrated for three hours and run for 12 - 14 hours with the mobile phase (heptane saturated with 96% methanol) in the 96% methanol: heptane (1 : 1) chromatography system (12). The standard strips are cut out of the air-dried chromatograms and the areas of etiocholanolone, androsterone, and dehydroepiandrosterone standards are developed by dipping each strip in Zimmermann Reagent and gently heating the saturated strip until a purple band develops. Three by five cm. areas corresponding to the standard etiocholanolone bands and equal sized paper blank strips above the origin and below the androsterone band are cut out of the extract strip. The strips are eluted with four ml. of 75% methanol : 25% chloroform for three hours at

37° C. The eluate is air-dried taken up in 1.0 ml. of ethanol, and a 0.1 ml. portion is taken to determine radioactivity.

Quantitation of Etiocholanolone:

The remaining 0.9 ml. eluate is again air-dried and .04 ml. of freshly prepared dinitrophenylhydrazine reagent (4 mg. of 2, 4-dinitrophenylhydrazine in two ml. of absolute ethanol acidified with .05 ml. of concentrated HCl) is added to each tube (12). The reagent and etiocholanolone eluate are allowed to react for at least 12 hours at room temperature. A standard curve is made with three and five microgram dilutions of etiocholanolone. The standard curve conforms to Beer's Law (14).

After the steroid and phenylhydrazine reaction come to completion, 1.0 ml. of distilled water and 0.5 ml. of fresh Benedict's reagent is added to each tube. The tubes are heated in a water bath at 100° C. for ten minutes. The mixture is extracted with 1.0 ml. of chloroform by agitating with a mechanical vibrating device for one minute. The separated chloroform layer is pipetted into Beckman microcuvettes. Optical density readings are made on the standard curve, the extracts from the plasma samples, paper blanks, and the reagent blanks, in a Beckman DU Spectrophotometer at a wavelength of 368 mμ using microcuvettes with a 1.0 cm. light path.

Isotopic Recovery:

The 0.1 ml. portions of the eluates, as well as the reference amount of tritium-labelled etiocholanolone initially added, are air-dried in isotope counting vials. The tritium-labelled etiocholanolone is counted in a TMC-Phosphor Scintillation counter (efficiency for counting tritium-labelled etiocholanolone 13%, Standard Error 7 $\pm$ 2%). The chemical recovery of etiocholanolone is then corrected for the losses detected by isotope dilution (15).

Recovery Experiments:

Recovery experiments were performed by extracting 50 ml. portions of stored pooled human plasma to which three or five micrograms of etiocholanolone standard as well as isotopic etiocholanolone (approximately 10,000 counts per minute) were added. Control samples from the same plasma pool were also analyzed.

Results

Recovery Experiments:

The results of the chemical and isotopic recovery experiments are listed in Table 1. No measurable amount of etiocholanolone was recovered from the control samples taken from the same plasma pool. The overall recovery attained by correcting the chemical recovery for losses determined by the isotope dilution was about 80%.

Etiocholanolone in the Plasma of Dogs and Rabbits:

The results of the analyses of the dog plasma samples for unconjugated etiocholanolone are listed in Table 2. Each dog tended to have a constant level of etiocholanolone regardless of the experimental conditions. High concentrations were measured in the plasma samples of two of three animals. In each experiment, the control sample concentration was the same or greater than the corresponding experimental samples.

The results of the analyses of five post-vaccine experimental rabbit samples and four control specimens (three matched rabbit controls and one pooled specimen) are listed in Table 3. Each of the experimental samples had varying levels of etiocholanolone whereas only the pooled control sample had a low concentration of etiocholanolone. The male rabbits 01, and especially 03, had much higher levels than the female animals.

It should be noted that the volume of the control

specimens from rabbits 00 and 01 were not quite half the volume of the experimental specimens. Since the probability of detecting microgram quantities of steroid increases with the volume of the plasma sample, it might be suspected that etiocholanolone was not detected in 00 and 01 because of the small volume of the control samples. Thus, the control samples 21, 22, and 23 were pooled to obviate this discrepancy. The results show higher etiocholanolone levels in all five of the experimental samples than in the pooled control sample.

Identification:

The measured dinitrophenylhydrazones were pooled, air-dried, and taken up in redistilled petroleum ether : benzene (1 : 1 by volume). This extract was then placed on a micro-column 12.5 cm. long by 0.3 cm. wide containing 1000 - 1100 mg. of 5% alumina that had been washed with petroleum ether : benzene (1 : 1). The extract was eluted from the column with 5 ml. of benzene : chloroform (4 : 1) and collected in 0.5 ml. fractions. A standard etiocholanolone-2, 4-dinitrophenyl-hydrazone was run in a similar manner on the same size column and exhibited the same mobility as the pooled dinitrophenyl-hydrazine extract. The extract was again air-dired, taken up in petroleum ether : benzene (1 : 1), and applied to a micro-column containing 1000 - 1100 mg. of 3.9% alumina washed with benzene : petroleum ether (1 : 1). The extract was eluted with 6 ml. of the benzene : chloroform (4 : 1) and collected in 0.5 ml. fractions. The standard and measured plasma etiocholanolone-

dinitrophenylhydrazone extract had the same characteristic elution mobility in this system also. The Specific Activity (counts per minute per microgram) of the etiocholanolone-dinitrophenylhydrazone extract was 1930 after chromatography on 5% alumina and 1960 after chromatography of 3.9% alumina.

Discussion

The specificity of this method depends upon complete chromatographic separation of etiocholanolone from androsterone and dehydroepiandrosterone as well as from all of the other steroids present in the plasma extract. The identification of the pooled etiocholanolone-dinitrophenylhydrazone by its mobility compared to standard etiocholanolone-dinitrophenylhydrazone in two column chromatographic systems and maintainence of constant Specific Activity after two chemical isolations, substantiates the validity of the determination.

The results of the analyses of rabbit plasma show higher concentrations of etiocholanolone in the post-vaccine samples. Since a small number of animals were tested and the magnitude of the increase of etiocholanolone levels varied a great deal, it is difficult to establish that any characteristic response to the pyrogen took place.

The observation that etiocholanolone fails to produce a febrile response in the rabbit subjected to a standard pyrogen assay reduces the likelihood that the etiocholanolone present in the post-vaccine blood sample represents endogenous pyrogen (4).

Kappas has reported that the etiocholanolone injected into human subjects has a half-life of less than one hour and

is therefore not present in the blood at the time of the febrile response following its injection (4). Moreover, plasma from one of his febrile subjects did not produce a fever when injected into normal volunteers. In contrast to the delayed pyrogenic activity of etiocholanolone, endogenous pyrogen is found in greatest amounts in blood samples from animals at the height of the second phase of their febrile response to an endotoxin (9). The observations that the two pyrogens have different time-relationships to the febrile response which they induce makes it unlikely that the unconjugated etiocholanolone found in the plasma of febrile rabbits represents endogenous pyrogen.

The etiocholanolone concentrations in the experimental dogs were generally high and unaffected by the injection of endotoxins. The possibility that the pentobarbital anesthesia elevated the blood etiocholanolone levels in these dogs was not investigated.

The differences of the plasma etiocholanolone level in the dog and rabbit may have been a result of the small number of animals studied or a species difference in their response to the physiological stress imposed by the pyrogens.

The male animals had the highest levels of plasma unconjugated etiocholanolone. This included three dogs and two out of five rabbits. It is possible that the etiocholanolone present may have been of testicular, and not adrenal,

origin. The report of Kass that no 17-ketosteroid could be detected after chromatography of rabbit adrenal vein blood would support this conjecture (16).

Summary and Conclusions

A method for the quantitative determination of unconjugated etiocholanolone in plasma is presented. The material measured by this method was identified as etiocholanolone by comparison of its chromatographic mobility with standard etiocholanolone.

Plasma samples from the dog and rabbit were analyzed by this method before and after the injection of pyrogens administered to elicit the release of endogenous serum pyrogen. Unconjugated etiocholanolone was found in both control and experimental samples from the dog and rabbit. The plasma unconjugated etiocholanolone levels in the dog were unchanged after the injection of pyrogen, whereas the levels were generally elevated in the experimental samples from rabbits.

Since no characteristic increase in levels of unconjugated etiocholanolone occurred in dogs or rabbits after the injection of pyrogens, there is no evidence to support the hypothesis that unconjugated etiocholanolone acts as the endogenous serum pyrogen known to occur under these experimental conditions. Further studies are necessary to determine the significance of plasma levels of unconugated etiocholanolone in the dog and rabbit.

Table 1

ETIOCHOLANOLONE RECOVERIES

Number of Experiments	Micrograms of Etiocholanolone	Isotopic Recovery Mean % ± S.D.	Chemical Recovery Mean % ± S.D.	Corrected Recovery Mean % ± S.D.
4	3	73 ± 5.6	59 ± 4.0	81 ± 3.0
4	5	74 ± 5.0	59 ± 3.3	80 ± 4.0

Table 2

UNCONJUGATED ETIOCHOLANOLONE

IN DOG PLASMA

Animal	Experimental Conditions	Etiocholanolone micrograms per 100 ml. plasma
B	control	8.2
B-1	V-58	0
B-B-2	V-58	0
B-3	Pyromen	7.1
C	control	25.0
C-1	V-58	25.0
D	control	33.0
D-1	Pyromen	20.0
D-2	Pyromen	0
D-3	V-58	28.0
D-4	V-58	23.5

Table 3

UNCONJUGATED ETIOCHOLANOLONE

IN RABBIT PLASMA

Animal	Sex	Experimental Conditions	Etiocholanolone micrograms/100 ml. plasma	ml. plasma/ sample
00	female	control	0	28
00-1	female	NDV-14	3.2	66
01	male	control	0	29
01-1	male	NDV-14	8.0	77
02	male	control	0	31
02-1	male	NDV-14	23.0	38
21, 22, 23	females	pooled control	1.9	88
22-1	female	NDV-14	2.2	100
23-1	female	NDV-14	2.5	83

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